

Amendments to the Specification:

Please replace the paragraph at page 6, line 22 through page 7, line 17 with the following amended paragraph:

Zveg4 proteins can be prepared as fusion proteins comprising amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, an affinity tag, or a targeting polypeptide. For example, a zveg4 protein can be prepared as a fusion with an affinity tag to facilitate purification. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include, for example, a poly-histidine tract, protein A (Nilsson et al., *EMBO J.* 4:1075, 1985; Nilsson et al., *Methods Enzymol.* 198:3, 1991), glutathione S transferase (Smith and Johnson, *Gene* 67:31, 1988), a Glu-Glu affinity tag (Grussenmeyer et al., *Proc. Natl. Acad. Sci. USA* 82:7952-4, 1985), substance P, Flag™, FLAG™ peptide (Hopp et al., *Biotechnology* 6:1204-1210, 1988), streptavidin binding peptide, maltose binding protein (Guan et al., *Gene* 67:21-30, 1987), cellulose binding protein, thioredoxin, ubiquitin, T7 polymerase, or other antigenic epitope or binding domain. Fusion of zveg4 to, for example, maltose binding protein or glutathione S transferase can be used to improve yield in bacterial expression systems. In these instances the non-zveg4 portion of the fusion protein ordinarily will be removed prior to use. Separation of the zveg4 and non-zveg4 portions of the fusion protein is facilitated by providing a specific cleavage site between the two portions. Such methods are well known in the art. Zveg4 can also be fused to a targeting peptide, such as an antibody (including polyclonal antibodies, monoclonal antibodies, antigen-binding fragments thereof such as F(ab')₂ and Fab fragments, single chain antibodies, and the like) or other peptidic moiety that binds to a target tissue.

Please replace the paragraph at page 8, line 35 through page 9, line 30 with the following amended paragraph:

Zveg4 proteins, including full-length polypeptides, variant polypeptides, biologically active fragments, and fusion proteins, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells (including cultured cells of multicellular organisms). Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, and Ausubel et al., eds., *Current Protocols in Molecular Biology*, Green and Wiley and Sons, NY, 1993. In

general, a DNA sequence encoding a zveg4 polypeptide is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers. See, for example, WO 00/34474. Exemplary expression systems include yeasts, such as *Saccharomyces cerevisiae* (see, e.g., U.S. Patent No. 5,527,668) or *Pichia methanolica* (U.S. Patents Nos. 5,716,808, 5,736,383, 5,854,039, and 5,955,349); mammalian cells, such as baby hamster kidney (BHK) cells (ATCCTM No. CRL 1632 or No. CRL 10314), COS-1 cells (ATCCTM No. CRL 1650), COS-7 cells (ATCCTM No. CRL 1651), 293 cells (ATCCTM No. CRL 1573; Graham et al., *J. Gen. Virol.* 36:59-72, 1977) or Chinese hamster ovary cells (e.g. CHO-K1, ATCCTM No. CCL 61; or CHO DG44, Chasin et al., *Som. Cell. Molec. Genet.* 12:555, 1986); baculovirus (Luckow et al., *J. Virol.* 67:4566-4579, 1993; available in kit form (~~Bac-to-Bac~~ BAC-TO-BACTM kit; ~~Life Technologies~~ LIFE TECHNOLOGIESTM, Rockville, MD)); and bacterial cells (e.g., *E. coli*). Suitable cell lines are known in the art and available from public depositories such as the ~~American Type Culture Collection~~ AMERICAN TYPE CULTURE COLLECTIONTM, Manassas, VA.

Please replace the paragraph at page 14, lines 8-22 with the following amended paragraph:

Zveg4 was identified from the sequence of a clone from a human chronic myelogenous leukemia cell (K562) library by its homology to the VEGF family. Additional sequence was elucidated from a long sequence read of a clone from a pituitary library. An antisense expressed sequence tag (EST) for zveg4 was found, for which its 5' partner was identified. This 5' EST (EST448186; GenBank) appeared to contain the 5' untranslated sequence for zveg4. A primer was designed from EST448186 to close the gap in the sequence. 20 pm each of oligonucleotides ZC21,987 (SEQ ID NO:5) and ZC21,120 (SEQ ID NO:6) and 1.93 µg of a thyroid library were used in a PCR reaction with 5% DMSO and 1/10 volume of a commercial reagent (~~GC-Melt~~ GC-MELTTM; ~~Clontech~~ CLONTECHTM Laboratories, Inc., Palo Alto, CA). The reaction was run for 1 minute at 94°C; then 30 cycles of 94°C, 20 seconds; 67°C, 1 minute; then a final 5-minute incubation at 72°C. A resulting 833-bp product was sequenced and found to be a

zveg4 fragment containing the remainder of the coding sequence with an initiation MET codon, upstream stop codon, and 5' untranslated sequence. The composite sequence included an open reading frame of 1,110 bp (SEQ ID NO:1).

Please replace the paragraph at page 14, line 25 - page 15, line 4 with the following amended paragraph:

A partial mouse zveg4 sequence was obtained by probing a mouse genomic library (obtained from ~~Clontech~~ CLONTECH™ Laboratories, Inc.) with a 1,289 bp EcoRI human zveg4 restriction digest fragment containing the entire coding sequence. The probe was labeled with ³²P using a commercially available kit (~~Rediprime~~ REDIPRIME™ II random-prime labeling system; ~~Amersham Pharmacia~~ AMERSHAM PHARMACIA™, Buckinghamshire, England). Unincorporated radioactivity was removed using a commercially available push column (~~NueTrap~~ NUCTRAP® column; ~~Stratagene~~ STRATAGENE™, La Jolla, CA; see U.S. Patent No. 5,336,412). Twenty-four filter lifts were prehybridized overnight at 50°C in a hybridization solution (~~ExpressHyb~~ EXPRESSHYB™ Hybridization Solution; ~~Clontech~~ CLONTECH™ Laboratories, Inc.) containing 0.1 mg/ml salmon sperm DNA that had been boiled 5 minutes, then iced. Filters were hybridized overnight at 50°C in hybridization solution (~~ExpressHyb~~ EXPRESSHYB™) containing 1.0×10^6 cpm/ml zveg4 probe, 0.1 mg/ml salmon sperm DNA, and 0.5 µg/ml mouse cot-1 DNA that had been boiled 5 minutes, then iced. Filter lifts were washed in 2 x SSC, 0.1% SDS at room temperature for 2 hours, then the temperature was raised to 60°C for one hour. Overnight exposure at -80°C showed 7 putative primary hits.

Please replace the paragraph at page 15, lines 5-8 with the following amended paragraph:

Four of the primary hits were plated on a lawn of *E. coli* K802 cells (obtained from ~~Clontech~~ CLONTECH™ Laboratories, Inc.). Filter lifts were prepared and hybridized overnight with the human zveg4 probe. Two of the 4 primary putative hits that were tested came up positive.

Please replace the paragraph at page 15, lines 28-34 with the following amended paragraph:

A full-length cDNA clone was generated by a two-step ligation of fragments from the two clones. An EcoRI/HindIII 3' fragment was prepared from clone zveg4mpzp7x-6. The 528-bp fragment was gel-purified and ligated into a phagemid vector (~~pBluescript~~ PBLUESCRIPT® II KS(+); ~~Stratagene~~ STRATAGENE™) that had

been digested with EcoRI and HindIII. Three µg of the resulting construct was digested with 15 units of EcoRI. The linearized plasmid was purified and ligated with a 754-bp 5' EcoRI fragment from clone zveg4mpzp7x-7.

Please replace the paragraph at page 16, lines 2-20 with the following amended paragraph:

Recombinant human zveg4 having a carboxyl-terminal Glu-Glu affinity tag was produced in a baculovirus expression system according to conventional methods. The culture was harvested, and the cells were lysed with a solution of 0.02 M Tris-HCl, pH 8.3, 1 mM EDTA, 1 mM DTT, 1 mM 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (~~Pefabloc~~ PEFABLOC® SC; Boehringer-Mannheim), 0.5 µM aprotinin, 4 mM leupeptin, 4 mM E-64, 1% NP-40 at 4°C for 15 minutes on a rotator. The solution was centrifuged, and the supernatant was recovered. Twenty ml of extract was combined with 50 µl of anti-Glu-Glu antibody conjugated to derivatized agarose beads (~~Sepharose~~ SEPHAROSE®; ~~Amersham Pharmacia~~ AMERSHAM PHARMACIA™ Biotech Inc., Piscataway, NJ) in 50 µl buffer. The mixture was incubated on a rotator at 4°C overnight. The beads were recovered by centrifugation and washed 3 x 15 minutes at 4°C. Pellets were combined with sample buffer containing reducing agent and heated at 98°C for five minutes. The protein was analyzed by polyacrylamide gel electrophoresis under reducing conditions followed by western blotting on a PVDF membrane using an antibody to the affinity tag. Two bands were detected, one at $M_r \approx 49$ kD and the other at $M_r \approx 21$ kD. Sequence analysis showed the larger band to comprise two sequences, one beginning at Arg-19 of SEQ ID NO:2 and the other beginning at Asn-35 of SEQ ID NO:2. The asparagine residue appeared to have been deamidated to an aspartic acid. The smaller band began at Ser-250 of SEQ ID NO:2.

Please replace the paragraph at page 16, line 23 - page 17, line 9 with the following amended paragraph:

Recombinant amino-terminally Glu-Glu-tagged zveg4 growth factor domain with an amino-terminal Glu-Glu (EYMPME; SEQ ID NO:9) tag (zveg4-nee-GFD) produced from recombinant baculovirus-infected insect cells was purified from the conditioned media by a combination of cation-exchange chromatography, antibody affinity chromatography, and size-exclusion chromatography. 28-liter cultures were harvested, and the media were filtered using a 0.45 µm filter. Filtered medium (pH 7.0, conductivity 9 mS) was directly loaded onto a 25-ml cation exchange column (~~Peros~~ POROS® 50 HS; PerSeptive Biosystems, Framingham, MA). The column was washed with ten column volumes (cv) of PBS, and the bound protein was eluted with a gradient

of 20-100% of 750 mM NaCl in PBS (Buffer B) for 15 cv followed by 5 cv of 100% Buffer B at 5 ml/min. Five-ml fractions were collected. Samples from the column were analyzed by SDS-PAGE with silver staining and western blotting for the presence of zveg4-nee-GFD. Zveg4-nee-GFD-containing fractions were pooled and loaded onto an 8-ml anti-Glu-Glu antibody column and eluted with 50 ml of 0.5 mg/ml EYMPTD (SEQ ID NO:10) peptide (obtained from Princeton Biomolecules Corporation, Langhorne, PA) in PBS. One-ml fractions were pooled and concentrated to 4 ml using a ~~Biomax~~ BIOMAXTM -5 concentrator (~~Millipore~~ MILLIPORETM Corp., Bedford, MA) and loaded onto a 16 x 1000 mm gel filtration column (~~Sephaeryl~~ SEPHACRYLTM S-100 HR; ~~Amersham-Pharmacia~~ AMERSHAM PHARMACIATM Biotech, Piscataway, NJ) at 1.5 ml/minute. Five-ml fractions containing purified zveg4-nee-GFD were pooled, filtered through a 0.2 μ m filter, aliquoted into 100 μ l aliquots, and frozen at -80°C. The concentration of the final purified protein was determined by BCA assay (Pierce Chemical Co., Rockford, IL) to be 0.4 mg/ml, and the yield was calculated to be 8.4 mg.

Please replace the paragraph at page 17, lines 10-32 with the following amended paragraph:

Recombinant zveg4-nee-GFD was analyzed by SDS-PAGE (~~Nupage~~ NUPAGETM 4-12% gel; ~~Novex~~ NOVEXTM, San Diego, CA) with silver staining (~~FASTsilver~~ FASTSILVERTM, Geno Technology, Inc., Maplewood, MO) and Western blotting using antibodies to the peptide tag. Conditioned media or purified protein was electrophoresed using an electrophoresis mini-cell (~~XCell-II~~ XCELL IITM mini-cell; ~~Novex~~ NOVEXTM) and transferred to nitrocellulose (0.2 μ m; ~~Novex~~ NOVEXTM) at room temperature using a blot module (~~XCell-II~~ XCELL IITM; ~~Novex~~ NOVEXTM) with stirring according to directions provided in the instrument manual. The transfer was run at 500 mA for one hour in a buffer containing 25 mM Tris base, 200 mM glycine, and 20% methanol. The filters were then blocked with 10% non-fat dry milk in PBS for 10 minutes at room temperature. The nitrocellulose was quickly rinsed, then the mouse anti-peptide primary antibody (diluted 1:1000 in PBS containing 2.5% non-fat dry milk) was added. The blots were incubated for two hours at room temperature or overnight at 4°C with gentle shaking. Following the incubation, blots were washed three times for 10 minutes each in PBS, then labeled with a secondary antibody (goat anti-mouse IgG conjugated to horseradish peroxidase) diluted 1:1000 in PBS containing 2.5% non-fat dry milk, and the blots were incubated for two hours at room temperature with gentle shaking. The blots were then washed three times, 10 minutes each, in PBS, then quickly rinsed with H₂O. The blots were developed using commercially available chemiluminescent substrate reagents (~~SuperSignal~~ SUPERSIGNAL[®] ULTRA reagents 1

and 2 mixed 1:1; reagents obtained from Pierce Chemical Co.), and the signal was captured using image analysis software (~~Lumi-Imager~~ LUMI IMAGER™ Lumi Analyst 3.0; Boehringer Mannheim GmbH, Germany) for times ranging from 10 seconds to 5 minutes or as necessary.

Please replace the paragraph at page 18, lines 19-25 with the following amended paragraph:

Zvefg4-ccc-containing fractions were pooled and concentrated to 3.8 ml by filtration using a ~~Biomax~~ BIOMAX™ -5 concentrator (~~Millipore~~ MILLIPORE™ Corp.), and loaded onto a 16 x 1000 mm gel filtration column (~~Sephaeryl~~ SEPHACRYL™ S-200 HR; ~~Amersham Pharmacia~~ AMERSHAM PHARMACIA™ Biotech). The fractions containing purified zvefg4-ccc were pooled, filtered through a 0.2 µm filter, aliquoted into 100 µl each, and frozen at -80°C. The concentration of the final purified protein was determined by colorimetric assay (BCA assay reagents; Pierce Chemical Co.) and HPLC-amino acid analysis.

Please replace the paragraph at page 18, line 32 - page 19, line 17 with the following amended paragraph:

To prepare adenovirus vectors, the protein coding region of zvefg4 is amplified by PCR using primers that add FseI and AscI restriction sites at the 5' and 3' termini, respectively. PCR primers are used with a template containing the full-length zvefg4 cDNA in a PCR reaction as follows: incubation at 95°C for 5 minutes; followed by 15 cycles at 95°C for 1 min., 58°C for 1 min., and 72°C for 1.5 min.; followed by 72°C for 7 min.; followed by a 4°C soak. The reaction products are loaded onto a 1.2 % (low melt) (~~SeaPlaque~~ SEAPLAQUE GTG™; FMC, Rockland, ME) gel in TAE buffer. The zvefg4 PCR product is excised from the gel and purified using a spin column containing a silica gel membrane (~~QIAquick~~ QIAQUICK™ Gel Extraction Kit; ~~Qiagen~~ QIAGEN™, Inc., Valencia, CA) as per kit instructions. The zvefg4 product is then digested, phenol/chloroform extracted, EtOH precipitated, and rehydrated in 20ml TE (Tris/EDTA pH 8). The zvefg4 fragment is then ligated into the cloning sites of the transgenic vector pTG12-8. Vector pTG12-8 was derived from p2999B4 (Palmiter et al., *Mol. Cell Biol.* 13:5266-5275, 1993) by insertion of a rat insulin II intron (ca. 200 bp) and polylinker (Fse I/Pme I/Asc I) into the Nru I site. The vector comprises a mouse metallothionein (MT-1) promoter (ca. 750 bp) and human growth hormone (hGH) untranslated region and polyadenylation signal (ca. 650 bp) flanked by 10 kb of MT-1 5' flanking sequence and 7 kb of MT-1 3' flanking sequence. The construct is transformed into *E. coli* host cells (~~Electromax~~ ELECTROMAX DH10B™ cells; obtained from Life

Technologies LIFE TECHNOLOGIES™, Inc., Gaithersburg, MD) by electroporation. Clones containing zveg4 DNA are identified by restriction analysis. A positive clone is confirmed by direct sequencing.

Please replace the paragraph at page 19, line 23 - page 20, line 5 with the following amended paragraph:

The zveg4 cDNA is cloned into the FseI-AscI sites of a modified pAdTrack CMV (He et al., *Proc. Natl. Acad. Sci. USA* 95:2509-2514, 1998). This construct contains a green fluorescent protein (GFP) marker gene. The CMV promoter driving GFP expression has been replaced with the SV40 promoter, and the SV40 polyadenylation signal has been replaced with the human growth hormone polyadenylation signal. In addition, the native polylinker has been replaced with FseI, EcoRV, and AscI sites. This modified form of pAdTrack CMV is named pZyTrack. Ligation is performed using a DNA ligation and screening kit (~~Fast-Link~~ FAST-LINK™; Epicentre Technologies, Madison, WI). In order to linearize the plasmid, approximately 5 µg of the pZyTrack zveg4 plasmid is digested with PmeI. Approximately 1 µg of the linearized plasmid is cotransformed with 200 ng of supercoiled pAdEasy (He et al., *ibid.*) into BJ5183 cells. The co-transformation is done using a Bio-Rad Gene Pulser at 2.5kV, 200 ohms and 25 µF. The entire co-transformation is plated on 4 LB plates containing 25 µg/ml kanamycin. The smallest colonies are picked and expanded in LB/kanamycin, and recombinant adenovirus DNA is identified by standard DNA miniprep procedures. Digestion of the recombinant adenovirus DNA with FseI and AscI confirms the presence of zveg4 DNA. The recombinant adenovirus miniprep DNA is transformed into *E. coli* DH10B competent cells, and DNA is prepared therefrom.

Please replace the paragraph at page page 20, lines 6-26 with the following amended paragraph:

Approximately 5 µg of recombinant adenoviral DNA is digested with PacI enzyme (New England Biolabs) for 3 hours at 37°C in a reaction volume of 100 µl containing 20-30U of PacI. The digested DNA is extracted twice with an equal volume of phenol/chloroform and precipitated with ethanol. The DNA pellet is resuspended in 10 µl distilled water. A T25 flask of QBI-293A cells (Quantum Biotechnologies, Inc., Montreal, Canada), inoculated the day before and grown to 60-70% confluence, are transfected with the PacI digested DNA. The PacI-digested DNA is diluted up to a total volume of 50 µl with sterile HBS (150 mM NaCl, 20 mM HEPES). In a separate tube, 20 µl of 1mg/ml N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium methylsulfate (DOTAP; Boehringer Mannheim) is diluted to a total volume of 100 µl

with HBS. The DNA is added to the DOTAP, mixed gently by pipeting up and down, and left at room temperature for 15 minutes. The media is removed from the 293A cells and washed with 5 ml serum-free MEM-alpha (~~Life Technologies~~ LIFE TECHNOLOGIES™, Gaithersburg, MD) containing 1 mM sodium pyruvate (~~Life Technologies~~ LIFE TECHNOLOGIES™), 0.1 mM MEM non-essential amino acids (Life Technologies) and 25 mM HEPES buffer (~~Life Technologies~~ LIFE TECHNOLOGIES™). 5 ml of serum-free MEM is added, and the cells are held at 37°C. The DNA/lipid mixture is added drop-wise to the flask, mixed gently, and incubated at 37°C for 4 hours. After 4 hours the media containing the DNA/lipid mixture is aspirated off and replaced with 5 ml complete MEM containing 5% fetal bovine serum. The transfected cells are monitored for GFP expression and formation of foci (viral plaques).

Please replace the paragraph at page page 21, lines 26-35 with the following amended paragraph:

The virus recovered from the gradient includes a large amount of CsCl, which must be removed before it can be used on cells. ~~Pharmacia~~ PHARMACIA™ PD-10 columns prepacked with ~~Sephadex~~ SEPHADEX® G-25M (~~Pharmacia~~ PHARMACIA™) are used to desalt the virus preparation. The column is equilibrated with 20 ml of PBS. The virus is loaded and allowed to run into the column. 5 ml of PBS is added to the column, and fractions of 8-10 drops collected. The optical density of a 1:50 dilution of each fraction is determined at 260 nm on a spectrophotometer, and a clear absorbance peak is identified. Peak fractions are pooled, and the optical density (OD) of a 1:25 dilution is determined. OD is converted into virus concentration using the formula $(OD \text{ at } 260nm)(25)(1.1 \times 10^{12}) = \text{virions/ml}$.